

# APT102, a Novel ADPase, Cooperates With Aspirin to Disrupt Bone Metastasis in Mice

Özge Uluçkan,<sup>1</sup> Mark C. Eagleton,<sup>1</sup> Desiree H. Floyd,<sup>1</sup> Elizabeth A. Morgan,<sup>1</sup> Angela C. Hirbe,<sup>1</sup> Matthew Kramer,<sup>1</sup> Nikki Dowland,<sup>1</sup> Julie L. Prior,<sup>2</sup> David Piwnica-Worms,<sup>2</sup> Soon Seog Jeong,<sup>3</sup> Ridong Chen,<sup>3</sup> and Katherine Weillbaeher<sup>1\*</sup>

<sup>1</sup>Departments of Medicine and Cell Biology and Physiology, Division of Oncology, Washington University School of Medicine, St. Louis, Missouri 63110

<sup>2</sup>Molecular Imaging Center, Mallinckrodt Institute of Radiology, and Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

<sup>3</sup>APT Therapeutics, 893 North Warson Road, St. Louis, Missouri 63141

**Abstract** Platelets contribute to the development of metastasis, the most common cause of mortality in cancer patients, but the precise role that anti-platelet drugs play in cancer treatment is not defined. Metastatic tumor cells can produce platelet  $\alpha$ IIb $\beta$ 3 activators, such as ADP and thromboxane A<sub>2</sub> (TXA<sub>2</sub>). Inhibitors of platelet  $\beta$ 3 integrins decrease bone metastases in mice but are associated with significant bleeding. We examined the role of a novel soluble apyrase/ADPase, APT102, and an inhibitor of TXA<sub>2</sub> synthesis, acetylsalicylic acid (aspirin or ASA), in mouse models of experimental bone metastases. We found that treatment with ASA and APT102 in combination (ASA + APT102), but not either drug alone, significantly decreased breast cancer and melanoma bone metastases in mice with fewer bleeding complications than observed with  $\alpha$ IIb $\beta$ 3 inhibition. ASA + APT102 diminished tumor cell induced platelet aggregation but did not directly alter tumor cell viability. Notably, APT102 + ASA treatment did not affect initial tumor cell distribution and similar results were observed in  $\beta$ 3<sup>-/-</sup> mice. These results show that treatment with ASA + APT102 decreases bone metastases without significant bleeding complications. Anti-platelet drugs such as ASA + APT102 could be valuable experimental tools for studying the role of platelet activation in metastasis as well as a therapeutic option for the prevention of bone metastases. *J. Cell. Biochem.* 104: 1311–1323, 2008. © 2008 Wiley-Liss, Inc.

**Key words:** ADPase; aspirin; bone metastasis; platelets

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Ö. Uluçkan and M.C. Eagleton contributed equally to this work.

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\*Correspondence to: Katherine Weillbaeher, Departments of Medicine and Cell Biology and Physiology, Division of Oncology, Washington University School of Medicine, 660 S. Euclid, Box 8069, St. Louis, MO 63110.  
E-mail: [kweillbae@im.wustl.edu](mailto:kweillbae@im.wustl.edu)

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Bone metastases, the most common type of metastasis for patients with breast and prostate cancer, can result in significant bone loss, fractures, pain, hypercalcemia, and spinal cord compression. Osteoclast mediated bone resorption is a critical component of bone metastasis and can promote tumor growth in bone [Guise, 2000; Mundy, 2002; Hirbe et al., 2006]. Anti-resorptive therapy significantly decreases skeletal complications of cancer and is considered standard of care for patients with bone metastases; however, 50% of patients with bone metastases develop new bone metastases, new skeletal complications and disease progression [Hortobagyi et al., 1996; Roodman, 2004].

Pre-clinical and clinical data support a facilitating role for platelets, another non-malignant host factor, in the metastatic process [Pruemer, 2005; Castelli and Porro, 2006; Zwicker et al., 2007]. As cancer cells travel from a primary site to a distant metastatic site, they

co-exist with platelets in thrombi located throughout the organs and circulatory system of patients with metastatic cancer [Gouin-Thibault et al., 2001; Billroth, 2003]. More than 50% of metastatic breast cancer patients have tumor cells detectable in blood termed circulating tumor cells (CTC). CTC number and response to cytotoxic therapy have been shown to predict progression-free and overall survival [Wiedswang et al., 2004; Braun et al., 2005].

Metastatic tumor cell lines induce platelet adhesion and aggregation *in vitro* through the production of numerous platelet activators such as ADP, thromboxane, thrombin, and von Willebrand factor [Lerner et al., 1983; Gasic, 1984; Karpatkin et al., 1988; Nierodzik et al., 1996; Bakewell et al., 2003; Camerer et al., 2004; Medina et al., 2006; Witz, 2006]. Indeed, the metastatic potential of tumor cell lines is markedly diminished in mice with defective platelet aggregation [Karpatkin et al., 1988; Amirkhosravi et al., 2002; Francis and Amirkhosravi, 2002; Bakewell et al., 2003; Camerer et al., 2004; Hu et al., 2004; Shi et al., 2004; Palumbo et al., 2005]. Moreover, tumor cells engineered to respond to platelet-derived lysophosphatidic acid (LPA) have enhanced bone metastatic potential in mice [Boucharaba et al., 2004]. However, few studies in humans have directly tested the efficacy of anti-platelet agents to inhibit or decrease metastatic tumor growth [Akl et al., 2007; Tagalakis et al., 2007].

Platelet  $\beta_3$  integrins have been implicated in the development of bone metastases [Phillips et al., 1991; Smyth et al., 2001; Bakewell et al., 2003; Camerer et al., 2004]. Mice lacking the  $\beta_3$  integrin are protected from tumor-associated bone osteolysis [Bakewell et al., 2003] and inhibition of platelet specific integrin  $\alpha_{IIb}\beta_3$  decreases the incidence of bone metastasis in mice.  $\alpha_{IIb}\beta_3$  plays a central role in the initiation of arterial thrombosis and platelet aggregation [Phillips et al., 1991; Smyth et al., 2001; Bakewell et al., 2003; Camerer et al., 2004]. When resting platelets are activated via ADP, thrombin or epinephrine,  $\alpha_{IIb}\beta_3$  is activated, thereby enabling binding of its ligand, fibrinogen [Schwartz et al., 1995; Takagi and Springer, 2002]. ADP binding to its G-protein-coupled-receptors (P2Y<sub>12</sub> and P2Y<sub>1</sub>) not only induces  $\alpha_{IIb}\beta_3$  activation, but also rapidly initiates platelet production of another platelet activator, thromboxane A<sub>2</sub> (TXA<sub>2</sub>) [Murugappan et al., 2004; Maxwell et al., 2006].

Metastatic tumor cells can produce ADP and TXA<sub>2</sub> among other factors and can induce  $\beta_3$  integrin activation and platelet aggregation [Boukerche et al., 1994; Gately and Li, 2004]. Aspirin (acetylsalicylic acid or ASA) inhibits cyclooxygenase (COX), a critical enzyme in TXA<sub>2</sub> synthesis [Awtry and Loscalzo, 2000; Catella-Lawson and Crofford, 2001; Wang and Dubois, 2006], and effectively decreases platelet aggregation. ASA treatment moderately decreases tumor cell-induced platelet aggregation (TCIPA) *in vitro*, however when used as a single agent, does not affect pulmonary metastases in mice [Camerer et al., 2004]. ASA has also been used as a chemoprevention agent to prevent the development of invasive cancer from pre-cancerous lesions [Dube et al., 2007], but its role on established tumors and during bone metastasis has not been defined.

APT102 is a novel form of the naturally occurring human apyrase/ADPase, CD39 [Medina et al., 2006]. Apyrases promote the breakdown of extracellular ATP and ADP by catalyzing the removal of the gamma phosphate from ATP and the beta phosphate from ADP [Jin et al., 2005; Marcus et al., 2005]. CD39<sup>-/-</sup> mice have increased bleeding times and deficient platelet aggregation. APT102 is a soluble, mutated form of the CD39 protein that cleaves ADP more potently than endogenous CD39, and has also been shown to prevent Caco-2 human colon cancer cell induced platelet aggregation *in vitro* [Medina et al., 2006].

We hypothesized that treating mice with ASA + APT102 in combination would lead to decreased tumor burden in bone and tumor-associated bone loss. We examined the effect of these agents on tumor cell dissemination in two murine bone metastasis models, in which tumor cell lines expressing firefly luciferase and bioluminescence imaging (BLI) are used to assess real-time tumor burden with increased sensitivity at multiple time points.

## MATERIALS AND METHODS

### Animal Studies

C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were used for B16 melanoma studies, and BALB/c mice (Taconic, Hudson, NY) and  $\beta_3$  integrin<sup>-/-</sup> mice on BALB/c background (a generous gift from Dr. Robin Anderson, Peter MacCallum Cancer Institute, East Melbourne,

Victoria, Australia [Sloan et al., 2006]) were used for 4T1 breast cancer studies. Mice were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine, St. Louis, MO. The Animal Studies Committee of Washington University School of Medicine approved all experiments.

### Cell Lines

The osteolytic B16-F10 murine C57BL/6 melanoma cell line was obtained from ATCC (Manassas, VA) and modified to express firefly luciferase (B16-FL) [Hirbe et al., 2006]. The osteolytic 4T1 murine BALB/c breast carcinoma cell line was modified to express GFP and firefly luciferase (4T1-GFP-FL) [Hirbe et al., 2006]. Cell lines were cultured in  $\alpha$ -MEM with 10% fetal calf serum.

### Expression, Purification, and Pharmacokinetics of APT102

APT102 is a soluble mutant form of human CD39L3, generated by removal of the N-terminal 44 amino acids and the C-terminal 43 amino acids. Two point mutations, R67G and T69R, were introduced into the CD39L3 coding sequence as previously described to significantly enhance ADPase activity [Medina et al., 2006]. The mutant gene, APT102, was cloned into an expression plasmid and secreted APT102 protein was purified as described from conditioned medium of transfected HEK293T or CHO cells [Medina et al., 2006].

Pharmacokinetic studies were conducted in rats where single bolus APT102 was intravenously injected (0.75 mg/kg,  $n = 3$  per time-point). Serum samples were examined for both ADPase and ATPase activities. The ADPase and ATPase activities of APT102 were estimated by ADP and ATP hydrolysis assays using malachite green [Baykov et al., 1988]. Enzymatic analysis was initiated by the addition of 5  $\mu$ l of the enzyme to 495  $\mu$ l of a mixture containing 50 mM Tris-HCl (pH 7.4), 8 mM CaCl<sub>2</sub>, and various concentrations of ADP or ATP. Following 30-min incubation at 37°C, 50  $\mu$ l of the reaction solution was mixed with 900  $\mu$ l of 50 mM Tris-HCl (pH 7.4) and 50  $\mu$ l of the malachite working solution. The inorganic phosphate released from the ADP or ATP reacts with the malachite working solution, resulting in a green color. Since the enzymatic activity of

apyrases is proportional to the amount of the released inorganic phosphate, apyrase activity can be measured by monitoring the absorbance at 630 nm using an Agilent 8453 UV-Visible spectrophotometer (Agilent, Palo Alto, CA). The kinetics of the enzymatic reaction was determined by measuring the time course of the color development at a wavelength of 630 nm.

The experimental data best fit biphasic exponential curves for either enzyme activity. The distribution phase half-life ( $T^{1/2}$ ) of APT102 was calculated to be 40 min and 50% of apyrase activity was cleared from the circulation during this phase. After 24 h the enzyme activity was still significantly elevated above baseline (100 vs. 10 pmol min<sup>-1</sup>) with a plasma elimination  $T^{1/2}$  of 20 h. In mice, APT102 (3.1 mg/kg,  $n = 3$  per time point), had a comparable  $T^{1/2}$  and the dosing interval was chosen to be every 12 h by intravenous injection.

Ex vivo effects of APT102 on ADP-induced platelet aggregation in PRP from APT102 treated mice were also evaluated. Ten minutes after administration of a single bolus of APT102 there was greater than 90% inhibition of ADP-induced platelet aggregation. Inhibition was long lasting, and still significant 6 h after dosing.

### Platelet Aggregometry

Platelet aggregation was measured by light aggregometry according to previous methods [Palumbo et al., 2005]. Mouse blood was isolated via cardiac puncture in the presence 5 U/ml heparin. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation of whole blood as previously described [Palumbo et al., 2005]. PRP ( $1 \times 10^9$ /ml) was placed in a whole blood ionized calcium lumi-aggregometer (Chronolog Corp., Havertown, PA). Light transmission increases as platelets form into aggregates. TCIPA was initiated by the addition of B16-FL cells ( $10^6$  cells/ml) at time 0 min, and the reaction was monitored and analyzed by the Aggro-link data processing system (Chronolog) for 6 min [Jurasz et al., 2001]. To study the effect of ASA and a potato apyrase (Sigma A6410, St. Louis, MO), platelets were pre-incubated with ASA (10 mM), apyrase (5 U), or ASA+apyrase for 3 min before initiation of aggregation [Alonso-Escolano et al., 2004]. Collagen at 5  $\mu$ g/ml and 5  $\mu$ M ADP were used as control agonists.

### Platelet-Tumor Adhesion Assay

Wildtype and  $\beta_3$ -/- platelets were incubated in a 96-well plate at 37°C for 1 h. Plates were then treated with 1% BSA in PBS for 1 h. The non-adherent cells were washed off 6× with PBS.  $10^5$  B16-FL cells were added and incubated for 1 h. The remaining non-adherent cells were then washed off 6× with PBS and binding was measured by Cyquant Cell Proliferation ELISA Kit (Molecular Probes, Eugene, OR).

### Bleeding Time Measurements

Bleeding time measurements were performed as described previously with minor modifications [Broze et al., 2001]. Briefly, mice were immobilized and a small transaxial incision was made on the tail. The tail was then immersed in 37°C PBS and blood flow was visually observed and timed for up to 5 min.

### MTT Assay

B16-FL or 4T1-GFP-FL cells were plated and allowed to adhere for 24 h in 96-well plates. Vehicle, 0.1 mg/ml ASA, 0.003 mg/ml APT102 or the combination was then added to the media. MTT assay was performed according to manufacturer's instruction (Sigma).

### Administration of Pharmacologic Agents

Mice were treated with 30 mg/kg ASA via oral gavage and/or 3.15 mg/kg APT102 via tail vein injection 30 min prior to all experiments in Figures 1–3 unless otherwise indicated. For long-term metastasis experiments in Figures 1–3, ASA and/or APT102 were additionally administered every 12 h post-tumor cell inoculation for a total of 2½ days. For the experiments in Figure 4, ASA + APT102 were given 1 h prior to tumor cell inoculation as a single dose.

### Bone Metastasis Studies

For intra-cardiac (i.c.) injections, operators were blinded to treatment group. Mice were anesthetized with a xylazine-ketamine cocktail and inoculated intra-cardially with  $1 \times 10^5$  B16-FL or 4T1-GFP-FL cells in 0.05 ml PBS as previously described [Bakewell et al., 2003; Hirbe et al., 2006]. In vivo BLI was performed on days 2 and 9 for 4T1-GFP-FL and day 9 for B16-FL. Each in vivo study was repeated at least once to confirm result significance and treatment groups from separate experiments

were pooled. Mice that received 4T1-GFP-FL were euthanized and underwent necropsy on day 9 (for histomorphometric analyses) or day 14 (for radiographic analyses) after tumor cell inoculation. Mice that received B16-FL were euthanized and underwent necropsy on day 12.

### Ultrasound-Guided Intra-Cardiac Delivery of 4T1-GFP-FL Cells

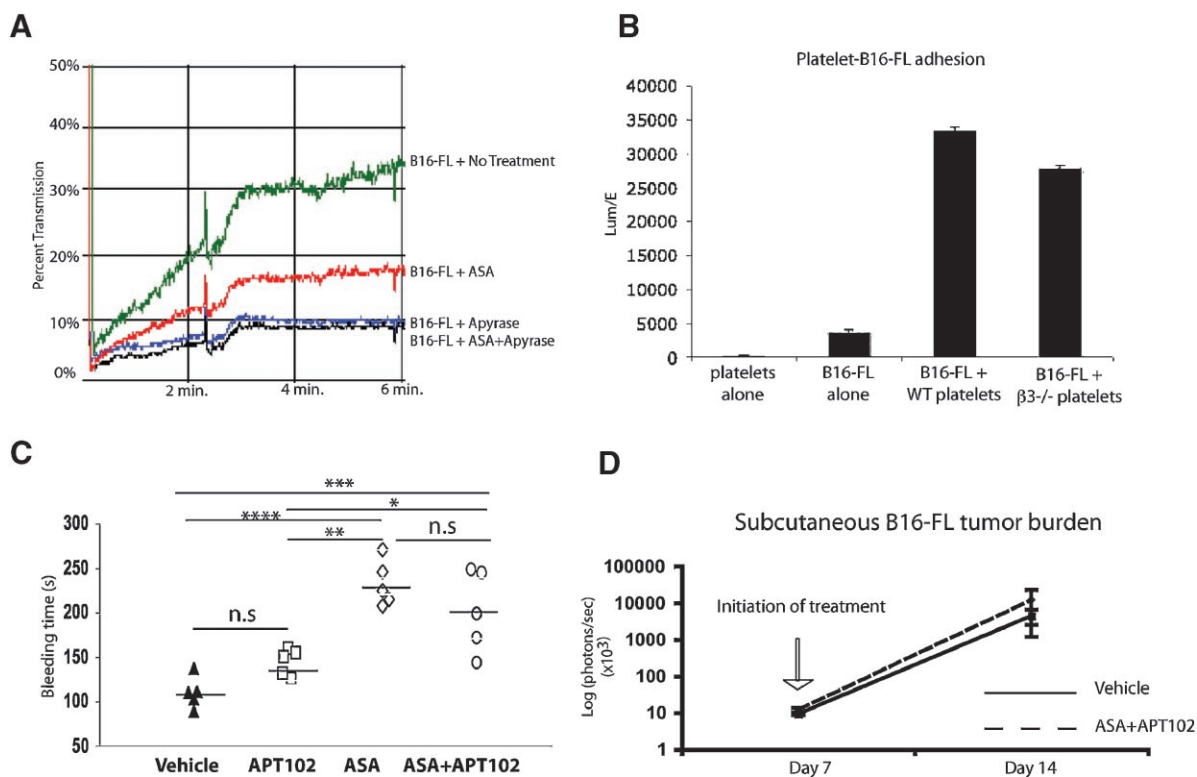
Ultrasound-guided injection of 4T1-GFP-FL cells into the left ventricular chamber of the bleeding-prone  $\beta_3$ -/- mice was performed using a Vevo770 Ultrasound System (Visual Sonics, Inc, Toronto, Canada). Mice were anesthetized with gaseous isoflurane and secured on an imaging platform in supine position, and the anterior chest was shaved. Ultrasound images of the left ventricle were obtained with a mechanical ultrasound transducer operating at 30 MHz imaging frequency. The cell suspension was injected using a needle secured to the injection arm of the Vevo770 imaging platform. The needle was then advanced through the apex of the left ventricle into the mid cavity, and the cell suspension was injected. After the needle was retracted, mice were monitored for evidence of hemorrhage into the pericardial or pleural space.

### In Vivo Bioluminescence Imaging (BLI)

Mice were injected intraperitoneally with 150 mg/kg of D-luciferin in PBS 10 min prior to imaging. Imaging was performed using a charge coupled device (CCD) camera (IVIS 100, Xenogen, Alameda, CA; exposure times 1 s, 10 s, 1 min or 5 min, binning 8, FOV 15 cm, f/stop 1, no filter) in collaboration with the Molecular Imaging Center (Washington University in St Louis, MO). Mice were anesthetized by isoflurane (2% vaporized in O<sub>2</sub>) and C57BL/6 mice were shaved to minimize attenuation of light by pigmented hair. Total photon flux (photons/s) was measured while the operator was blinded to treatment group from a fixed region-of-interest (ROI) over the tibia/femur area, and mandible using Living Image and IgorPro software (Wavemetrics, Portland, OR) as previously described [Hirbe et al., 2006].

### Subcutaneous (s.c) Tumor Cell Implantation

Mice were anesthetized with a xylazine-ketamine cocktail and  $2 \times 10^5$  4T1-GFP-FL cells were injected into the flank s.c. Mice were



**Fig. 1.** Aspirin and apyrase decreased tumor cell-induced platelet aggregation and increased bleeding times but did not affect local tumor growth. **A:** Platelets were pre-incubated with ASA (10 mM), potato apyrase (5 U), the combination or nothing for 3-min prior to addition of B16-FL cells. All four aggregometry tracings of treated platelets were flat prior to tumor cell addition. B16-FL cells were added to the platelets at time 0 min and aggregation was measured for 6 min. ASA, apyrase, and ASA + apyrase treatment decreased B16-FL tumor cell-induced platelet aggregation. **B:** B16-FL adhesion to a lawn of wild type and  $\beta_3^{-/-}$ -platelets or BSA (B16-FL alone) was measured by Cytquant Elisa Kit. B16-FL cells adhered to both wildtype and

$\beta_3^{-/-}$  platelets in vitro. **C:** Bleeding times (s = seconds) were measured in C57BL/6 mice treated 30 min prior with vehicle (n = 5), APT102 (n = 5), ASA (n = 5), or ASA + APT102 (n = 5). The treatment groups are denoted on the bottom of the graph. ASA and ASA + APT102 pretreatment elevated bleeding times compared to vehicle (\* $P = 0.0061$ , \*\* $P = 0.0002$ , \*\*\* $P = 0.0001$ , \*\*\*\* $P < 0.0001$ ). **D:** 4T1-GFP-FL s.c. tumors were established in Balb/c mice for 7 days. ASA + APT102 (n = 5) or vehicle (n = 5) was then administered and BLI was performed at days 7 (pre-treatment) and 14. No significant differences in s.c. tumor burden were observed between vehicle and ASA + APT102 ( $P = 0.23$ ).

treated with vehicle or ASA + APT102 at day 7 post-tumor cell inoculation and treatment continued every 12 h for 2½ days. BLI imaging was performed on day 7 (immediately before drug administration) and day 14. The operator was blinded to treatment group.

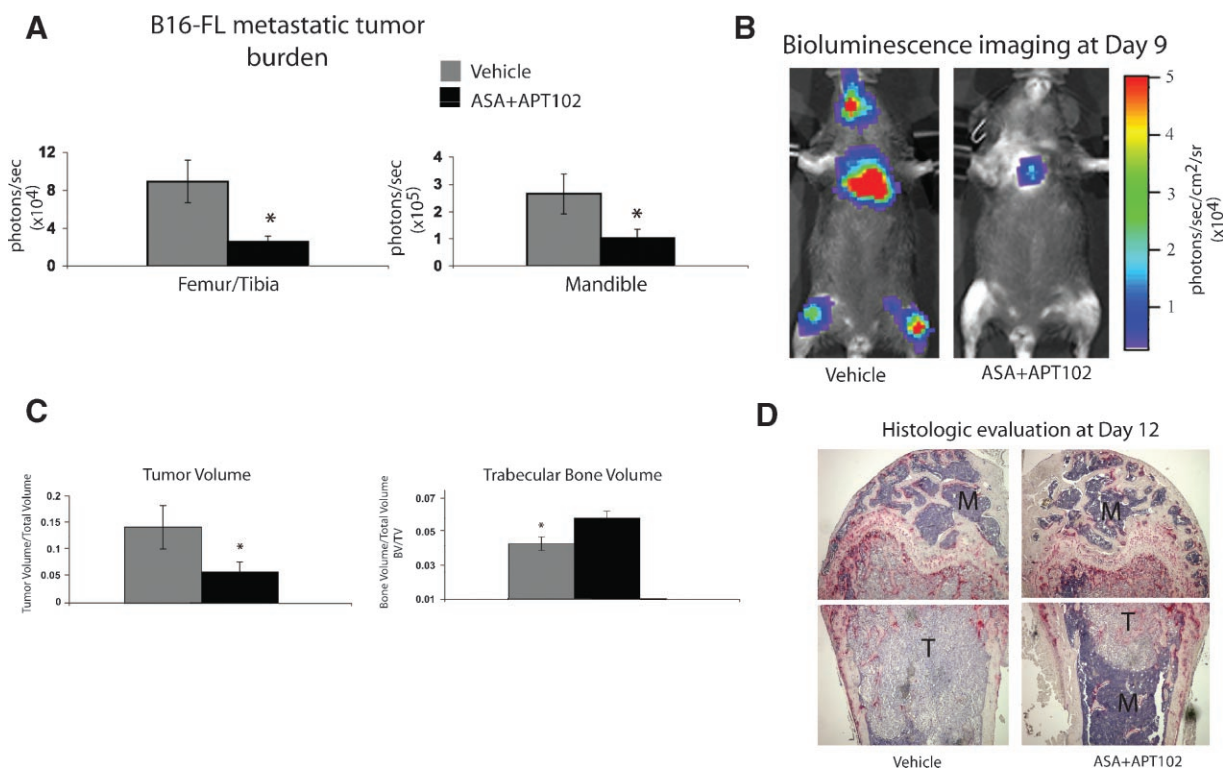
#### Quantification of Luciferase Activity in Organ Lysates

Wildtype BALB/c mice were treated with ASA + APT102 or vehicle as described above 1 h prior to 4T1-GFP-FL inoculation. Untreated  $\beta_3^{+/-}$  and  $\beta_3^{-/-}$  mice also underwent i.c. tumor cell inoculation under ultrasound guidance. One hour after tumor cell inoculation, vehicle-treated, ASA + APT102-treated,  $\beta_3^{+/-}$  and  $\beta_3^{-/-}$  mice were sacrificed and lungs, liver,

and bones were isolated and snap frozen. The frozen tissues were then weighed and homogenized with the appropriate amount of Glo Lysis Buffer (Promega, Madison, WI). For each gram of tissue, 875  $\mu$ l of Glo Lysis Buffer was added. The homogenate was vortexed and centrifuged. BLI was performed (IVIS, Xenogen Systems) on 50  $\mu$ l of the lysate supernatant in black-sided 96-well plates using the Bright-Glo Luciferase Assay System (Promega).

#### Bone Histomorphometry

Mouse femurs and tibiae were fixed in formalin and decalcified in 14% EDTA. Paraffin embedded sections were stained for tartrate resistant acid phosphatase (TRAP). Trabecular bone volume (BV/TV) and osteoclast perimeter



**Fig. 2.** Aspirin and APT102 in combination significantly decreased skeletal metastatic tumor burden in mice. C57BL/6 mice were treated with either vehicle or ASA + APT102 30-min prior to i.c. inoculation of B16-FL tumor cells, and drug treatments continued for 2½ days. Nine days later, BLI was performed and skeletal tumor burden was measured in femurs/tibiae and mandible in a consistent region of interest (ROI). **A:** B16-FL skeletal metastatic tumor burden as measured by BLI at day 9 was significantly decreased in the femur/tibia ( $P=0.002$ ) and mandible ( $P=0.03$ ) of mice treated with ASA + APT102 ( $n=20$ ) compared to vehicle ( $n=14$ ). **B:** Representative images

of BLI at day 9 are shown. **C:** Tumor volume and trabecular bone volume were measured by histomorphometric analysis on paraffin-embedded femurs of mice sacrificed 12 days after B16-FL inoculation (ASA + APT102 treated bones  $n=40$ , vehicle bones  $n=28$ ). Tumor volume was decreased in femurs of mice treated with ASA + APT102 compared to vehicle ( $P=0.04$ ). Trabecular bone volume was significantly increased in ASA + APT102-treated mice compared to vehicle ( $P=0.02$ ). **D:** Representative histological sections of femurs are depicted. T = Tumor, M = Marrow.

(OCPm/TbBPM) were measured from 120 to 1,080  $\mu\text{m}$  distal to the growth plate and tumor area/total area was measured from 120 to 4,800  $\mu\text{m}$  from the growth plate using Bioquant Osteo (Bioquant Image Analysis Corporation, Nashville TN) blinded to treatment group [Hirbe et al., 2006].

#### Statistical Analysis

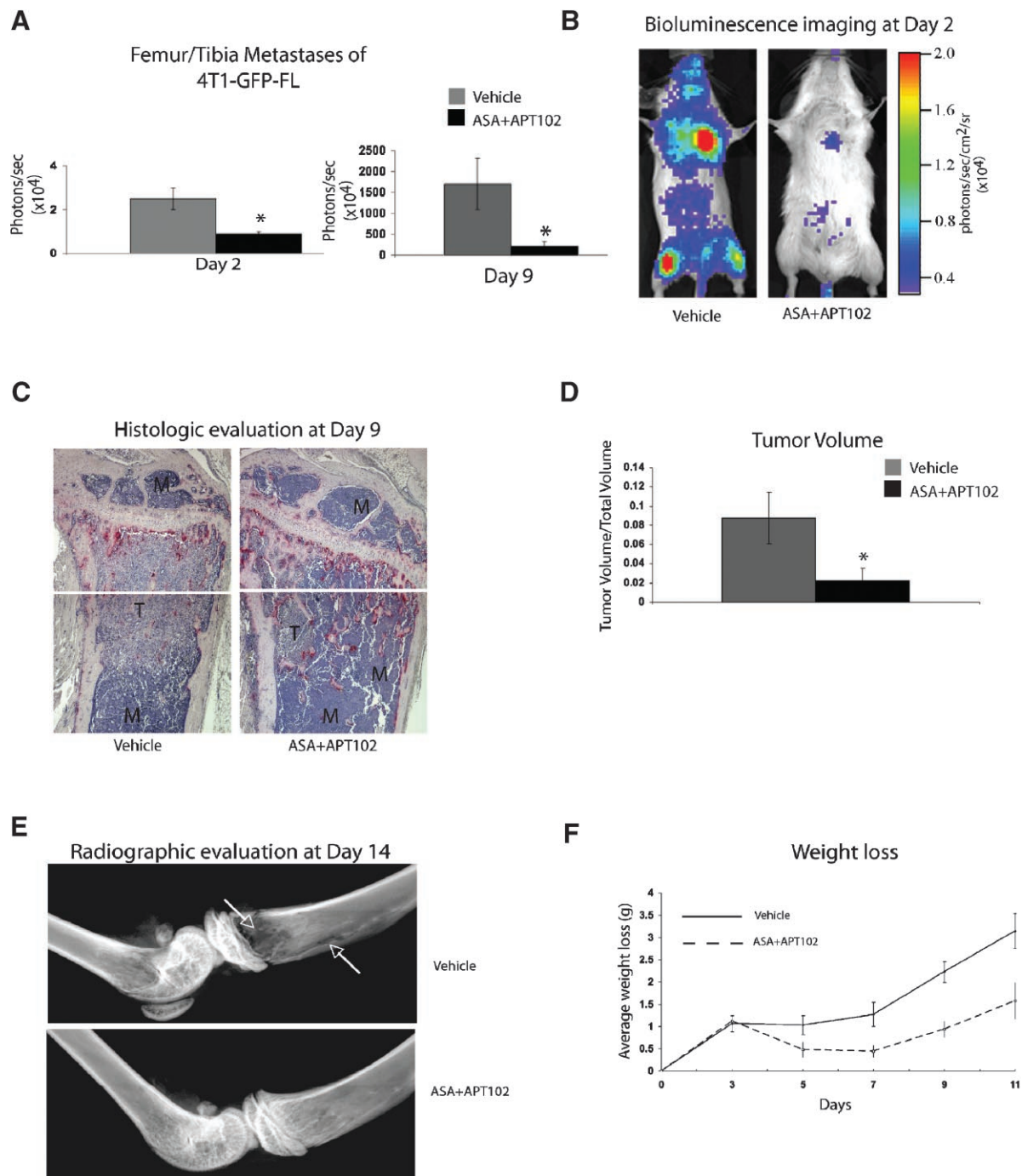
For experiments where two populations were compared to each other, group mean values were compared by two-tailed student  $t$ -test. In calculating two-tailed significance levels for equality of means, equal variances were assumed for the two populations. For the weight loss analysis in Figure 3, a non-parametric analog to a  $t$ -test is used, as the distributions of weights by day are reasonably symmetrical but clearly not gaussian. For the bleeding time

analysis in Figure 1 and MTT analysis in Supplemental Figure 1, where more than two populations are present, a mixed linear model was used to determine  $P$  values.

## RESULTS

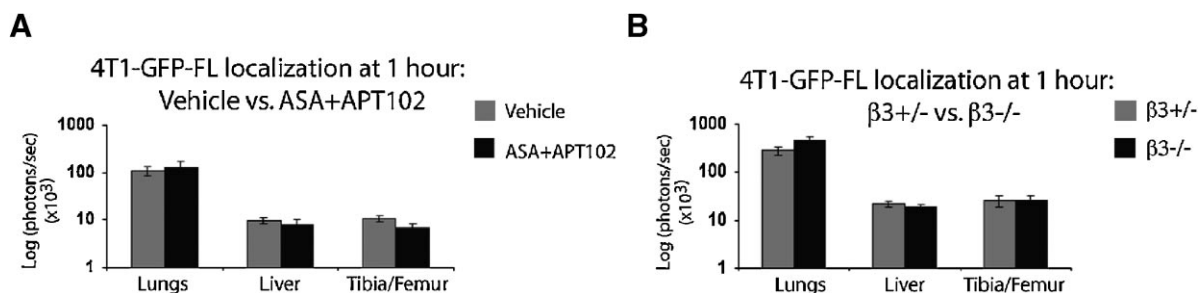
### Aspirin and Apyrase Decreased Tumor Cell-Induced Platelet Aggregation and Increased Bleeding Times but Did Not Affect Local Tumor Growth

We had previously found that inhibition of platelet  $\beta_3$  integrins blocks B16 tumor cell induced platelet aggregation in vitro [Bakewell et al., 2003]. Cancer cells induce platelet aggregation and secrete the platelet activators ADP and  $\text{TXA}_2$  [Grignani and Jamieson, 1988; Boukerche et al., 1994; Gately and Li, 2004]. We evaluated the ability of  $\text{TXA}_2$  inhibitor, ASA,



**Fig. 3.** Aspirin and APT102 in combination significantly decreased metastatic tumor burden in a second murine tumor model. BALB/c mice were treated with either vehicle or ASA + APT102 30-min prior to i.c. inoculation of 4T1-GFP-FL cells, and drug treatments continued for 2 $\frac{1}{2}$  days. BLI was performed at days 2 and 9 after tumor inoculation and skeletal tumor burden was measured in a consistent region of interest (ROI). **A:** 4T1-GFP-FL metastatic tumor burden is significantly decreased in the femur/tibia (day 2,  $P=0.01$ ; day 9,  $P=0.02$ ) of mice treated with ASA + APT102 ( $n=15$ ) compared to control ( $n=15$ ). **B:** Representative images of BLI at day 2. **C:** Representative histological sections of tibia. T=Tumor, M=Marrow. **D:** Tumor volume was measured by histomorphometric analysis

on paraffin-embedded femurs of mice sacrificed 9 days after tumor inoculation. Tumor volume was decreased in femurs of mice treated with ASA + APT102 compared to vehicle ( $P=0.04$ ). (ASA + APT102 treated bones  $n=30$ , vehicle treated bones  $n=30$ .) **E:** Representative radiographic images of femur/tibia taken at day 14. Arrows show areas of tumor-associated osteolysis. **F:** Serial weights were performed on mice starting at day 0 prior to tumor inoculation for 11 days. Average weight loss is plotted in grams. Vehicle treated mice lost significantly more weight after tumor inoculation compared to ASA + APT102 treated mice (vehicle  $n=8$ , ASA + APT102  $n=5$ , D0-D11  $P=0.04$ ).



**Fig. 4.** Tumor cell dissemination was not altered after ASA + APT102 treatment or in  $\beta_3^{-/-}$  mice. **A:** Wildtype BALB/c mice were treated with vehicle (n = 5) or ASA + APT102 (n = 5) 1-h prior to i.c. inoculation of 4T1-GFP-FL cells. One hour after tumor cell inoculation, organs were harvested, lysates were prepared, and luciferase activity was measured. At 1 h, 4T1-GFP-FL cell dissemination to all measured organs was not statistically different in ASA + APT102 and vehicle-treated mice (lungs

$P=0.17$ , liver  $P=0.44$ , tibia/femur  $P=0.95$ ). **B:** Platelet-defective  $\beta_3^{-/-}$  mice (n = 5) and normal  $\beta_3^{+/-}$  mice (n = 5) received i.c. inoculation of 4T1-GFP-FL tumor cells; 1-h post-inoculation, organs were harvested, lysates were prepared, and luciferase activity was measured. At 1 h, 4T1-GFP-FL cell dissemination to all measured organs was equivalent in  $\beta_3^{-/-}$  and  $\beta_3^{+/-}$  mice (lungs  $P=0.72$ , liver  $P=0.58$ , tibia/femur  $P=0.15$ ).

and ADP hydrolyzing agent, potato apyrase, to impair TCIPA. TCIPA was measured in the presence or absence of ASA, apyrase or the combination in vitro (Fig. 1A). Platelets isolated from wildtype C57BL/6 mice were incubated with ASA, apyrase, or ASA + apyrase, and aggregation was induced by addition of B16-FL murine osteolytic melanoma tumor cells. Apyrase treatment reduced B16-FL-induced platelet aggregation by 69% compared to control, while inhibition of platelet aggregation by ASA was more modest. ASA + apyrase treatment reduced platelet aggregation to a similar degree as apyrase treatment alone.

Tumor cells not only induce platelet aggregation, but also platelet adhesion [Karparkin et al., 1988]. To determine if platelet  $\beta_3$  integrins are necessary for tumor adhesion to platelets, B16-FL cells were incubated with wildtype or  $\beta_3^{-/-}$  platelet lawns in vitro and adherent tumor cells were quantitated. B16-FL cells adhered to both wildtype and  $\beta_3^{-/-}$  platelets to a similar extent indicating that the  $\beta_3$  integrin was not required for tumor-platelet adhesion (Fig. 1B).

Pharmacokinetic studies in mice showed that a single dose of APT102, soluble mutant form of the human apyrase CD39L3, led to more than 90% inhibition of ADP-induced platelet aggregation by as early as 10 min. The impact of APT102 and ASA on in vivo basal platelet aggregation was quantitated by measurements of bleeding times. Mice were pretreated with ASA, APT102, or ASA + APT102 in combination (ASA + APT102), and standardized bleeding times were measured 30 min after drug administration. ASA administration at doses previ-

ously published in mice [Sugimoto et al., 2006], significantly increased bleeding times by an average of 64% compared to vehicle-treated controls whereas APT102 increased bleeding times modestly by 22% compared to vehicle-treated controls. ASA + APT102 increased bleeding times to a similar degree as ASA treatment alone (Fig. 1C).

Direct effects of ASA + APT102 administration on tumor growth in vivo were evaluated on subcutaneously (sc) implanted 4T1-GFP-FL tumors. 4T1-GFP-FL sc tumors were established for 7 days and BLI was performed at day 7, after which ASA + APT102 or vehicle were administered and tumor growth monitored for an additional 7 days. No significant differences in tumor burden as measured by BLI were observed in mice treated with ASA + APT102 (Fig. 1D). ASA, APT102, or ASA + APT102 had no significant effect on 4T1-GFP-FL and B16-FL viability in vitro as measured by the MTT assay (Supplemental Fig. 1). These results demonstrate that ASA and APT102 in combination decreased B16-FL TCIPA in vitro and increased bleeding times in vivo, but had little direct effect on tumor cell viability either in vitro or in vivo.

#### Aspirin and APT102 in Combination Significantly Decreased Skeletal Metastatic Tumor Burden in Mice

Mice defective in  $\alpha IIb\beta_3$ -mediated platelet aggregation are protected from bone metastasis [Bakewell et al., 2003]. We hypothesized that pharmacologic inhibition of platelet aggregation by ASA and APT102 in combination would



provide protection from metastasis. Wildtype C57BL/6 mice were treated with ASA and/or APT102 30-min prior to i.c. inoculation of B16-FL tumor cells, and drug treatments continued for 2<sup>1</sup>/<sub>2</sub> days. We had previously published that treatment for 2<sup>1</sup>/<sub>2</sub> days with an inhibitor of platelet  $\alpha$ IIb $\beta$ 3 integrin (ML464) starting at 30-min prior to tumor inoculation significantly decreased tumor metastasis in mice [Bakewell et al., 2003]. Tumor burden was assessed in real time by in vivo BLI 9 days after tumor cell inoculation and was quantitated histologically at the time of sacrifice at day 12. ASA and vehicle-treated mice had comparable levels of tumor luciferase activity in the femur/tibia and mandible 9 days after tumor cell inoculation. A similar result was seen in APT102-treated mice (Supplemental Fig. 2A,B). In contrast, compared to vehicle-treated controls, the combination of ASA + APT102 produced a significant reduction in luciferase activity in the femur/tibia and mandible 9 days after i.c. tumor cell inoculation (Fig. 2A,B). In addition, there was no increase in i.c. injection procedure mortality in vehicle and treated groups compared to the 50% procedure mortality from hemorrhage reported in the  $\beta$ 3 integrin deficient mice [Bakewell et al., 2003].

Histomorphometric analysis of the femoral bones of mice treated with ASA + APT102 confirmed decreased tumor volume compared to vehicle-treated controls at day 12 (Fig. 2C,D). Likewise, there was a significant reduction in trabecular bone loss in ASA + APT102-treated mice compared to vehicle controls (Fig. 2C,D) consistent with decreased osteolytic B16-FL tumor burden. Thus, treatment with ASA + APT102 decreased metastatic melanoma tumor burden in bone and bone loss.

#### **Aspirin and APT102 in Combination Significantly Decreased Metastatic Tumor Burden in a Murine Breast Cancer Bone Metastasis Model**

We examined the effect of ASA, APT102, and ASA + APT102 treatment on bone metastatic tumor burden following i.c. inoculation of the murine breast cancer cell line, 4T1-GFP-FL, in BALB/c mice to control for the generality of response from cell line to cell line. 4T1-GFP-FL tumor luciferase activity could be detected in bone as early as 2 days after tumor cell inoculation due to increased intrinsic luciferase expression. No detectable differences in meta-

static tumor burden were observed in mice treated with ASA alone or APT102 alone compared to vehicle treated controls (data not shown).

However, administration of ASA + APT102 in combination resulted in significantly decreased tumor burden in the femur/tibia (Fig. 3A,B) compared to vehicle controls at days 2 and 9 after tumor cell inoculation. Histologic/histomorphometric analysis of femur/tibia from mice sacrificed at day 9 confirmed that luciferase activity correlated with viable tumor formation (Fig. 3C,D). A cohort of ASA + APT102 and vehicle treated mice were observed for longer times and radiographic images were taken 14 days after tumor inoculation. Representative radiographic images demonstrate visibly decreased bone loss seen as decreased areas of radiolucency in the ASA + APT102 mice compared to vehicle controls (Fig. 3E). In addition, ASA + APT102 treated mice had significantly decreased tumor associated weight loss compared to vehicle controls (Fig. 3F). To evaluate whether the decrease in bone loss observed was a direct effect of ASA + APT102 on bone, non-tumor bearing mice were treated with ASA + APT102 for 2.5 days. There was no significant change in trabecular bone volume or significant changes in osteoclast perimeter compared to vehicle controls (Supplemental Fig. 2C,D) with drug treatment alone. Thus, treatment with ASA + APT102 decreased tumor associated weight loss and 4T1-GFP-FL metastatic tumor burden in bone.

#### **Tumor Cell Dissemination Is Intact in Mice With Defective Platelet Function**

The decreased bone tumor burden by ASA + APT102 treatment observed as early as 2 days after i.c. tumor inoculation led us to hypothesize that platelets are important for the initial stages of tumor dissemination to the metastatic organ. To test this hypothesis, we evaluated tumor cell burden 1 h after i.c. inoculation of 4T1-GFP-FL tumor cells in mice with either a genetic or a pharmacologically induced defect in platelet function. One hour after tumor cell inoculation lungs, liver and bones were harvested. Luciferase activity of the lysates was analyzed after being normalized for weight. Lysates were used in this experiment because in vivo real time BLI was not sensitive enough at this early time point.

Tumor burden in the lung, liver, and bones of mice treated with ASA + APT102 1-h prior to 4T1-GFP-FL i.c. inoculation was not significantly different from vehicle-treated controls as measured by luciferase activity (Fig. 4A). Likewise, no difference in initial 4T1-GFP-FL tumor cell dissemination to end organs was observed in the platelet-defective  $\beta 3^{-/-}$  mice compared to normal  $\beta 3^{+/-}$  littermates (Fig. 4B). Thus, tumor cell dissemination to metastatic sites including bone in the first hour was intact in mice with defective platelet aggregation.

### DISCUSSION

We demonstrated significant inhibition of bone tumor burden by the novel ADPase, APT102, in combination with aspirin (ASA), using two experimental bone metastasis models in separate strains of immunocompetent mice. These agents were not directly cytotoxic to tumor cells and neither ASA + APT102 treatment nor genetic loss of  $\beta 3$  integrin altered initial tumor cell distribution. Finally, we observed decreased tumor associated bone loss and decreased tumor associated weight loss from a 2 $\frac{1}{2}$ -day treatment course of ASA + APT102.

One important implication of these data is that combination rather than single-agent anti-platelet activation therapy may be required for the prevention of metastasis. We found that TCIPA was inhibited *in vitro* by apyrase alone. However, single agent apyrase APT102 did not result in a decrease in metastatic tumor burden *in vivo*. One possible explanation for this requirement for combination anti-platelet therapy is that tumor cells activate platelets through a variety of mechanisms [Lerner et al., 1983; Nierodzik et al., 1992; Dorsam et al., 2002; Camerer et al., 2004]. Combination therapy with ASA and the ADP receptor inhibitor, clopidogrel, has been shown to be superior to single agent therapy in the treatment of coronary artery and cerebral vascular disease [Peters et al., 2003]. Many patients with metastatic cancer have detectable CTC in their peripheral blood, and changes in the number of CTCs correlate with response to treatment and disease progression [Seronie-Vivien et al., 2001; Gaforio et al., 2003]. Our results show that combination ASA + APT102 therapy decreased tumor burden in bone after tumor cell inoculation into the circulation. We propose that ASA

and ADPase or  $\beta 3$  integrin inhibition could be evaluated in patients with tumor cells in circulation.

Interestingly, we found no difference in end organ localization of tumor cells in the platelet-defective  $\beta 3^{-/-}$  mice or ASA + APT102-treated mice at 1 h after tumor cell inoculation compared to controls, despite a marked difference in bone tumor burden 2 and 9 days later. This suggests that tumor cell localization, or the initial organ-specific homing, remained intact even though platelet activation had been inhibited. We cannot exclude the possibility that the luciferase activity observed in lung, liver, and bone lysates was a result of tumor cells circulating in the capillary bed of these organs. It could be possible that platelets do facilitate vessel engagement of tumor cells and migration to specific compartments within the metastatic organ, for example the stem cell niche in the bone marrow (an area rich in growth factors and extracellular calcium)[Adams et al., 2007]. We are currently investigating endosteal lodgment/adhesion of tumor cells in platelet-defective animals to test this hypothesis.

It has been suggested that platelets decrease metastasis by associating with tumor cells in the bloodstream and contributing self/HLA antigens that could repel attack from anti-tumor natural killer (NK) or immune cells in the metastatic site. Indeed, Palumbo et al. [2005] found that intact NK cell function was required for the anti-metastatic benefits observed in the platelet activation-defective  $G\alpha q^{-/-}$  mice. APT102 and ASA had the most significant effect on metastatic bone tumor burden, but we also observed some decreases in tumor burden in the lung. It is possible that the treatment with ASA and APT102 would affect all types of metastases but that the models used in these studies favor evaluation of bone metastases. Identifying and targeting non-thrombogenic platelet pathways in the metastatic process could result in therapies that would limit bleeding side effects while maintaining anti-metastatic properties.

Trabecular bone loss was not significantly altered after 2 $\frac{1}{2}$  days of ASA + APT102 treatment in non-tumor bearing mice. There was also no significant difference in osteoclast perimeter suggesting that short treatment of ASA + APT102 had no dramatic effect on osteoclasts *in vivo*. However, it is possible that ASA + APT102 treatment might exert some effect on

the osteoblasts changing bone formation thus bone turnover. We cannot rule out small effects of ASA + APT102 directly on bone cells but we believe that the magnitude of this change is likely not sufficient to account for the tumor phenotype observed. It is possible that the broader/systemic effect of apyrase treatment, combined with the anti-inflammatory effects of ASA treatment, could act directly on bone to suppress the growth of bone tumors. ADP receptors, such as P2Y1, exist in bone resorbing osteoclasts for example, and treatment of cultured osteoclasts with ADP enhances bone resorption [Hoebertz et al., 2001]. Loss of ADP receptor activation, such as P2Y1 in the bone microenvironment, could prevent bone resorption necessary for tumor growth in bone. Inflammation is well known to increase osteoclast resorption and bone loss [Haynes, 2007]. Tumors produce ADP, TXA<sub>2</sub>, and other molecules that recruit inflammatory cells, which in addition to recruiting and activating platelets, could contribute to increased osteoclastic resorption thereby creating a more favorable environment for tumor growth in bone. It has been shown that a platelet  $\beta$ 3 integrin antagonist decreased the growth of established bone tumors that had been engineered to overexpress the platelet LPA receptor [Boucharaba et al., 2004]. Thus, it is possible that administration with APT102 and ASA for longer times could have a more profound effect on tumor burden in bone and possibly on bone parameters independent of presence of tumor cells.

Pharmacologic targeting of the pathways through which tumor cells activate platelets could decrease or prevent metastatic tumor burden in bone. The use of platelet activation inhibitors in combination could provide a safe way to decrease or prevent the formation of new bone metastatic lesions in high-risk cancer patients, in particular, those with CTC.

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